Symposium

The Nature and Application of Biocontrol Microbes: *Bacillus* spp.

Formulation of *Bacillus* spp. for Biological Control of Plant Diseases

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ABSTRACT

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Maximizing the potential for successfully developing and deploying a biocontrol product begins with a carefully crafted microbial screening procedure, proceeds with developing mass production protocols that optimize product quantity and quality, and ends with devising a product formulation that preserves shelf-life, aids product delivery, and enhances bioactivity. Microbial selection procedures that require prospective biocontrol agents to possess both efficacy and amenability to production in liquid culture increase the likelihood of selecting agents with enhanced commercial development potential. Scale-up of biomass production procedures must optimize product quantity without compromise of product efficacy or amenability to stabilization and formulation. Formulation of *Bacillus* spp. for use against plant pathogens is an enormous topic in general terms but limited in published specifics regarding formulations used in commercially available products. Types of formulations include dry products such as wettable powders, dusts, and granules, and liquid products including cell suspensions in water, oils, and emulsions. Cells can also be microencapsulated. Considerations critical to designing successful formulations of microbial biomass are many fold and include preserving biomass viability during stabilization, drying, and rehydration; aiding biomass delivery, target coverage, and target adhesion; and enhancing biomass survival and efficacy after delivery to the target. Solutions to these formulation considerations will not necessarily be compatible. Data from several biocontrol systems including the use of *B. subtilis* OH 131.1 (NRRL B-30212) to reduce Fusarium head blight of wheat are used to illustrate many of these issues. Using our recently described assay for efficiently evaluating biomass production and formulation protocols, we demonstrate the effectiveness, in vitro, of UV protectant compounds lignin (PC 1307) and Blankophor BBH in reducing OH 131.1 morbidity when cells were exposed to UV light from artificial sunlight.

A successful formulation of a *Bacillus* spp. strain for use against plant disease is potentially more readily achieved than is a successful formulation of microbial biomass that does not produce a resistant life stage comparable to the *Bacillus* spore (12). The durability of the *Bacillus* spore (11) allows a formulation scientist to consider some processes that may be chemically, physically, or environmentally more severe and would therefore not be feasible for formulating less resistant microbial biomass. Despite this apparent advantage in formulating biomass of *Bacillus* spp., obtaining direction from the literature for conducting such research is difficult. The topic of formulations of *Bacillus* spp. is at once both expansive and limited when surveying the considerable literature on formulations of *B. thuringiensis* for insect biological control and the paucity of published specifics on formulations of *Bacillus* spp. for use against plant pathogens. Details of fermentation and formulation procedures as well as formulation composition are lacking for commercial *Bacillus*-based plant disease control products because this information can be difficult to patent (or to enforce the patent) and is therefore held as proprietary information by companies selling such products.

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Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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While an effort will be made to draw on the literature that covers the formulation of *Bacillus* spp. for control of plant diseases, the breadth of literature on the formulation of *Bacillus* spp. for the control of insect targets and microbes other than *Bacillus* spp. for reducing plant disease necessitates the inclusion of examples from this body of literature. Further information on the topic of formulating microbial pest control agents appears in recently published reviews (4,5,14,23,37).

In this article, we have divided topics regarding the formulation of *Bacillus* spp. into four main areas. The first three will be explored only briefly to provide background information and include definitions of formulation terminology, types of formulation materials, and an overview of *Bacillus*-based products that are active against plant diseases and available in the United States. The fourth topic, goals for maximizing the likelihood of obtaining successful formulations, will include discussion on preserving biomass viability during stabilization, drying, and rehydration; aiding biomass delivery, target coverage, and target adhesion; and enhancing biomass survival and efficacy after delivery to the target. A recently devised microtiter plate assay that can expedite the obtainment of some of these goals will be described.

Formulation terminology. Terminology utilized to describe formulations of microbial products for the control of pests has been thoroughly reviewed (19,30) and, therefore, will only be summarized briefly here. A formulated microbial product, for purposes of this paper, is defined as a product composed of biomass of a biocontrol agent and ingredients to improve the survival and effectiveness of the product. Formulations of microbial biomass can be liquid or dry. Liquid formulation products are also known as flowable or aqueous suspensions and consist of biomass suspensions in water, oils, or combinations of both (emulsions).

Dry formulation products include wettable powders, dusts, and granules. Wettable powders consist of dry inactive and active ingredients (biomass) intended to be applied as a suspension in liquid. Dusts are powder-like and consist of dry inactive and active ingredients to be applied dry, generally to seeds or foliage. Granules can be described as a free flowing, aggregated product composed of dry inactive and active ingredients. They can be applied directly to the target plant, in furrow, or in the case of water dispersible granules, mixed into water where the suspension of biomass and inactive ingredients are applied to targets as a spray. The term microencapsulation is also associated with formulation of microbial biomass and refers to surrounding small amounts of active ingredient with a protective inert layer. The layer can consist of any number of polymers and the encapsulation itself is generally achieved either chemically (43) or by air-drying (10) or spray-drying (42) liquid suspensions containing active and microencapsulating ingredients.

Formulation amendments. An enormous number of amendments have been utilized in experimental and commercial formulations of *Bacillus* spp. and other biocontrol agents. In turn, these amendments can be grouped into any number of amendment types. Generally, amendments can be grouped as either carriers (fillers, extenders) or amendments that improve the chemical, physical, or nutritional properties of the formulated biomass. A selection of amendment types along with a limited number of examples of each type are shown in Table 1. An extensive treatment of the topic can be found elsewhere (3). Experimental formulations of *Bacillus* spp. that have effectively reduced plant disease have included clays (28); peat and chitin (24,36); methylcellulose (29), Ca-alginate, alginate manucol, or carob (35); carboxymethyl cellulose, vegetable oil, and polyvinyl pyrrolidone (20); and peptone (13,35) and nutrient medium (41).

Biocontrol products that contain a *Bacillus* spp. active against plant disease. Selections of plant disease biocontrol products that contain a *Bacillus* spp. are presented in Table 2.

TABLE 1. Types of amendments and example materials for formulating *Bacillus* biomass

Amendment type	Examples
Liquid carriers	Vegetable oils
Mineral carriers	Kaolinite clay, diatomaceous earth
Organic carriers	Grain flours
Stabilizers	Lactose, sodium benzoate
Nutrients	Molasses, peptone
Binders	Gum arabic, carboxymethylcellulose
Desiccants	Silica gel, anhydrous salts
Thickeners	Xanthan gum
Surfactants	Tween 80
Dispersants	Microcrystalline cellulose
UV protectants	
Sunscreens	Oxybenzone
Optical brighteners	Blankophor BBH
Light blockers	Lignin (PC 1307)
Stickers	Pregelatinized corn flour

Types of formulations for these products include liquids (aqueous suspensions and flowables), wettable powders, and a dry flake. These products usually contain one but sometimes two strains of active ingredient. Information on the specific composition and production of formulations of commercial biocontrol agents is largely proprietary as are the specific protocols utilized for producing, stabilizing, and/or drying the *Bacillus* biomass used in these products. Product label information available from company websites gives limited specifics on the composition of the formulation while providing details on combinations of the formulated product with fungicides or an additional microbial strain (Table 2). Because information on producing, stabilizing, and/or drying the biomass of any biocontrol agent is, to a great extent, strain specific, the remainder of this paper will focus on providing guidance on conducting research designed to develop a successful formulation of *Bacillus* biomass.

Considerations in producing formulations of biomass of *Bacillus* spp. The process of formulating a biocontrol product is only one link in the chain of steps that must take place before a commercial biocontrol product proceeds to the marketplace. Other links in the chain include agent discovery, production, and stabilization with mandatory consideration of costs and obtaining regulatory approval taking place at each step. In many ways, decisions made during each of these steps will influence (positively or negatively) the level of success achieved in completing the other steps. With this in mind, we will consider three goals to achieve in formulating microbial biomass and some initial steps that should be considered in order to obtain these goals.

Preserve biomass viability during stabilization, drying, and rehydration. A crucial initial step toward preserving biomass viability during stabilization, drying, and rehydration is to optimize fermentation protocols for not only maximal total biomass output but, concomitantly, for maximal biomass efficacy and amenability to formulation. When producing biomass of *Bacillus* spp., in most instances fermentation protocols should be designed to maximize the production of efficacious spores (11) rather than vegetative cells. Fermentation environments and culture age influence the efficacy, stability, and desiccation tolerance of many biocontrol agents including spore forming fungi (16,27,31), yeasts (45), and bacteria (40). With the Fusarium head blight biocontrol agent *Cryptococcus nodaensis* OH 182.9 (45), modification of production medium carbon content, carbon to nitrogen ratio, and harvest time of liquid cultures resulted in differences in the recovery of freeze-dried cells of the agent that differed by 3 log units after storage for 15 days at room temperature. Without optimizing fermentation environments for the production of formulation-competent biomass, success in preserving biomass viability during stabilization, drying, and rehydration is likely to be limited. Consideration of the rate of drying and the final moisture content or, preferably, water activity (a_w = equilibrium relative humidity, a measure of the vapor pressure generated by the moisture present in a dried product) of the formulated product (15) also deserve careful research attention. In the case of stabilizing protein

TABLE 2. Selected *Bacillus*-based plant disease biocontrol products

Product name	Company	*Bacillus* component	Formulation type[z]	Primary target
Serenade	AgraQuest, Davis, CA	*B. subtilis* QST 713	WP, aqueous suspension	Fungi, bacteria on multiple vegetables, fruits
EcoGuard	Novozymes, Salem, VA	*B. licheniformis* SB3086	Flowable	*Sclerotinia homoeocarpa* on turf
Kodiak	Gustafson, Plano, TX	*B. subtilis* GB03	WP (Conc.), flowable	Fungi on cotton, large-seeded legumes, soybeans
Yield Shield	Gustafson	*B. pumillus* GB34	WP (Conc.)	Fungi on soybeans
BioYield	Gustafson	*B. amyloliquefaciens* GB99 + *B. subtilis* GB122	Dry flake	Fungi on bedding plants in potting mixes
Subtilex	Beker Underwood, Ames, IA	*B. subtilis* MBI600	WP (Conc.)	Fungi on cotton, large-seeded legumes, soybeans
Hi Stick L + Subtilex	Beker Underwood	*B. subtilis* MBI600 + rhizobium	Flowable	Fungi on soybeans, peanut

[z] WP (Conc.) = wettable powder concentrate.

products, there is considerable literature available in the field of pharmaceutical research (7) that can provide valuable guidance in conducting research on formulating microbial biomass.

Aid biomass delivery, target coverage, and target adhesion. A requisite step toward obtaining this formulation goal is to be aware of the physical and chemical environment on the application target because this knowledge will dictate the choice of wetting agent (surfactant) and/or sticker used in the formulation. Different formulations of *B. thuringiensis* affected droplet size spectrums, which in turn influenced target coverage (44). Delivery equipment that operates under conditions deleterious to inoculum survival, such as high shear, temperature, or extended storage in liquid under high CO_2 pressure (which can drastically lower spray pH), must be avoided. While wetting agents can have a negative impact on biomass viability, they can be advantageous to use in biomass formulations because they reduce surface tension and promote more uniform coverage on aerial plant parts with waxy surfaces (46). Adhesion of antagonist biomass to the plant target can also be addressed via formulation. In unique work with *B. thuringiensis*, McGuire and Shasha (25) used a pregelatinized flour formulation that promoted target adhesion even after multiple rain events. Pregelatinized flour was treated with high pressure steam to expand the starch molecule, converting it from a water insoluble to water soluble state. Pregelatinized starch in granular formulations of *B. thuringiensis* became hydrated when granules were applied to dew-covered leaves and retrograded to a water insoluble conformation. These granules were then adherent and resisted wash-off by rain due to being water insoluble.

Enhance biomass survival and efficacy after delivery to target. A key step in obtaining this formulation goal is to be aware of the ecology and biology of both the antagonist and the target pathogen. Formulations containing moisture retaining polymers (8) or nutrient amendments may be useful in enhancing biocontrol agent survival. But solutions to one formulation consideration will not necessarily be compatible with other formulation objectives. In our work with cryoprotectants and Fusarium head blight biocontrol agent *C. nodaensis* OH 182.9, we have discovered that the trisaccharide melezitose, while effective in enhancing the survival of freeze-dried cells of the biocontrol agent (34), also appears to enhance pathogenic activity of *Gibberella zeae* (D. A. Schisler, *unpublished data*). Catabolite repression due to carbon sources in formulations of *Bacillus* spp. could also have a negative effect by reducing antibiotic production and thereby temporarily nullify a potential mode of action of the formulated biocontrol agent (47). Mixtures of biocontrol strains can show enhanced levels of disease control compared with that of individual strains (6,18,33). Separate nutritional niches of mixture components, limited overlap of the physical environmental optima of mixture component strains (17,22), or heightened biocontrol activity of a mixture component due to the presence of a second mixture component (1) offer possible explanations of increased biocontrol success with formulations of microbial mixtures.

Despite taking precautions to enhance the survival of a biocontrol strain, severe environmental conditions may drastically limit biocontrol agent establishment on a host target site. In the case of attempting to protect aerial plant parts with biocontrol agents, a formulation solution could take the form of replacing lost biocontrol inoculum or protecting the inoculum initially delivered to the host surface from deleterious environmental conditions. While significant improvements still are needed, controlled release formulations of *Bacillus* spp. for insect control show considerable promise (9) in providing *Bacillus* spores and toxin crystals over time from one application event and represent a virtually unexplored area for *Bacillus*-based products for use in reducing plant diseases. UV protectants also offer promise in protecting cells delivered to aerial plant surfaces and are discussed in more detail below.

Microtiter plate assay for expediting the obtainment of formulation goals. When the goals for formulating *Bacillus* spp. biomass are considered together, it is clear that a daunting number of variables ideally should be experimentally considered and optimized to produce a maximally effective, formulated, *Bacillus*-based biocontrol product. Parameters to be optimized include the liquid culture production environment (including medium composition, C:N ratio, total carbon and nitrogen loading, pH, type and quantity of antifoam, aeration rate, stir rate, temperature, and incubation time, etc.), quantity and type of formulation amendments, methodology of producing a formulated product (use of continuous flow centrifugation, granulators, rotary drum vacuum filters, extruders, and mixers), methodology for drying biomass (numerous variations of air-, freeze-, spray-, and fluidized bed-drying), methodology for storing the dried biomass product (possible inclusion of antioxidants, desiccants or activated charcoal, relative humidity, temperature, and vacuum packing), packaging materials (impermeable or selectively gas permeable) and composition (nutrient, chemical, and physical), and temperature of the rehydration medium. While these studies collectively will always be labor intensive to complete, a microtiter plate-based methodology developed by Slininger and Schisler (38) (Fig. 1)

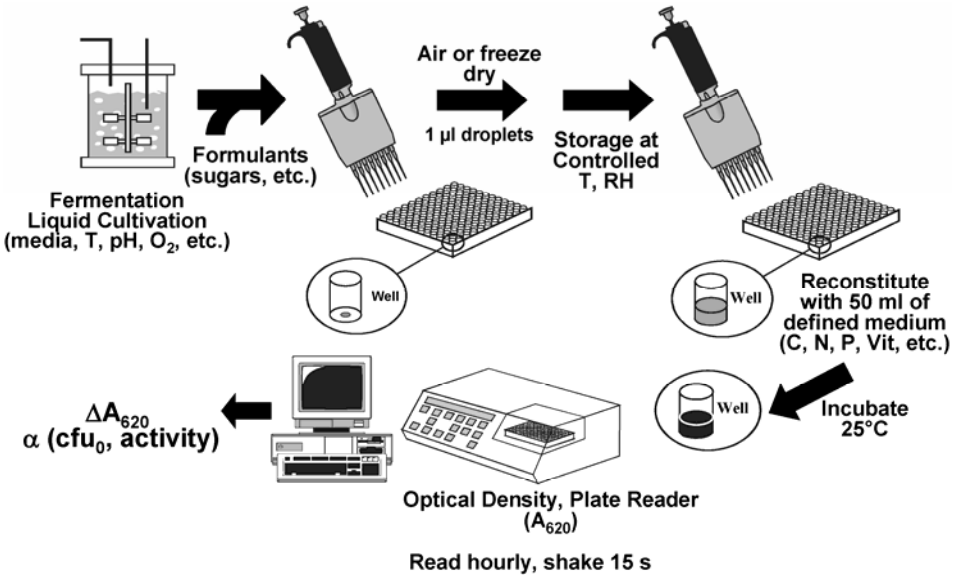

Fig. 1. Method to optimize factors that contribute to the survival of formulated biomass of *Bacillus* spp.

greatly lessens the time and effort required to reduce many of these process variables to a limited optimal range from which larger-scale, traditional laboratory assays can be used to confirm and fine tune these optima. Efficiency in scale is obtained using this technique by inoculating individual wells of 96 well microtiter plates with 1-µl droplets of experimental colonized broths and subjecting cells to formulation and drying treatments of the researcher's choosing. Storage conditions of the dried biomass can also be experimentally addressed as can conditions for rehydrating the biomass. Regardless of the experimental variables being studied, treatment effects on biomass survival are then evaluated by rehydrating test wells with a weak nutrient medium (that can also include rehydration medium treatments), incubating the plates under conditions favorable for microbial growth, and determining the change in A_{620} (absorbance at 620 nm) readings for individual wells over time. Wells with the highest A_{620} readings during logarithmic cell growth at a set time after rehydration indicate the treatments that fostered the highest number of metabolically active cell survivors.

The utility of this technique in identifying potentially useful UV protectant compounds was demonstrated using vegetative cells of Fusarium head blight antagonist *B. subtilus* OH 131.1. In previous research, we have demonstrated the potential of several antagonists including *B. subtilis* OH 131.1 to significantly reduce the severity of Fusarium head blight in field environments when biomass was produced in laboratory and pilot-scale quantities in liquid culture (21,32). Formulation research, in terms of UV protectants (2,26), may be crucial for the continued development of this biocontrol strain from bench discovery to biocontrol product. UV light can be devastating to the survival of microbial cells. A water soluble sodium salt of lignin (Westvaco PC 1307 experimental product, Charleston, SC) and the optical brightener Blankophor BBH (Sigma, Detroit, MI) have demonstrated UV protectant activity (26) but their effect on *Bacillus* spp. in general and OH 131.1 in particular was not known. The ability of these UV protectants to aid survival of dried cells of OH 131.1 exposed to artificial sunlight supplied from an xenon light source (Suntest Atlas CPS Solar Simulator, Heraeus DSET Laboratories Inc., Phoenix, AZ) was tested using the microtiter plate assay. Cells of antagonist OH 131.1 were grown in flasks containing a semi-defined liquid medium (39), harvested from 24 h growth cultures, combined or not with UV protectants, added as microliter droplets of formulated cells to 96 well microtiter plates (sterile Microtest flat bottom polystyrene, Becton Dickinson, Franklin Lakes, NJ), air dried for 1 h or not, and exposed or not to 6 h of artificial sunlight. Plates were placed on a refrigerated surface to maintain ambient temperatures within wells during exposure to artificial sunlight. A randomized complete block design was used in each of two separately conducted experiments. Data from both experiments were combined for statistical analysis. Cell counts at the time of introduction to microtiter plates were approximately 2×10^8 CFU/ml. Lignin concentrations were 0.2 and 0.3% (wt/vol), and BBH concentrations were 0.5, 1.0, and 2.0% (wt/vol). Wells were rehydrated with a weak growth medium and the growth of surviving cells was determined over time using a plate-reading spectrophotometer (MR5000; Dynatech Laboratories, Chantilly, VA) at 620 nm (Fig. 1). Predicted absorbance values (proportional to cell biomass concentration) were determined by weighted linear regression analysis of data obtained during logarithmic growth of cells (PROC REG, SAS PC Windows version 8.2). In vitro, lignin and BBH enhanced the survival of dried cells of OH 131.1 versus controls at all concentrations tested when cells were exposed to 6 h of UV as demonstrated by higher predicted absorbance values 30 h after rehydration (Table 3) in wells containing cells plus UV protectants. While 6 h of UV reduced the absorbance of control wells containing unprotected OH 131.1 after 30 h growth by about 51%, the absorbance in wells containing 0.2% lignin-protected OH 131.1 was only reduced by 14% when UV-treated and untreated wells were compared. The effect of UV protection was not necessarily improved as the concentration of protectants increased. UV protectants also enhanced OH 131.1 cell survival in wells that did not receive UV light, possibly due to protectants reducing cell viability losses otherwise caused by drying and rehydration stress (Table 3).

TABLE 3. Influence of UV protectants on dried 24-h *Bacillus* sp. strain OH 131.1 exposed to 0 or 6 h of artificial sunlight prior to rehydration of cells

Treatment	A_{620} 30 h after medium addition[x]		% A_{620} change due to 6-h UV exposure
	No UV	6-h UV	
OH 131.1 + 0.2% lignin[y]	0.148 a	0.131 a	–11.5
OH 131.1 + 0.3% lignin	0.140 b	0.120 b	–14.3
OH 131.1 control	0.082 e	0.040 e	–51.2
OH 131.1 + 0.5% Blankophor[z]	0.126 c	0.087 c	–31.0
OH 131.1 + 1.0% Blankophor	0.110 d	0.081 d	–26.4
OH 131.1 + 2.0% Blankophor	0.156 a	0.114 b	–26.9

[x] Within columns, mean values followed by the same letter are not significantly different (Duncan's multiple range test [$P < 0.05$]). Table data are predicted values based on linear regression analysis of observed absorbance at 620 nm (A_{620}) values during logarithmic cell growth.
[y] Experimental product Westvaco PC 1307, water soluble lignin.
[z] Optical brightener.

Conclusion. Formulation plays a significant role in determining the final efficacy of a *Bacillus*-based product, as do the processes of discovery, production, and stabilization of the biomass of the biocontrol agent. If a *Bacillus* strain reaches the stage of formulation development via poorly conceived or understood discovery, production, or stabilization protocols, the biocontrol and therefore commercial potential of this agent will be compromised. Effective formulations of biomass of *Bacillus* spp. for biocontrol of plant diseases are currently in the marketplace (Table 2), but this in no way indicates that improvements in formulation technology are not needed. The formulations of commercial products containing *B. thuringiensis* have undergone a wide range of transitions over the years including aqueous suspensions, wettable powders, oil flowables, insect pellet baits, dry flowable-fluidized bed agglomerates, and dry flowable low pressure extrusion granules (R. Venable, *personal communication*). With continued research, similar improvements in formulations can be anticipated for plant disease biocontrol products. Conducting this research in the public sector, not just the private sector, will greatly expedite progress in this critical area for advancing the successful incorporation of biocontrol products into the mainstream of tools for controlling plant diseases in production agriculture.

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